表4 LeRoyによる全身性強皮症病型分類

Diffuse cutaneous SSc (dcSSc)
1. Onset of Raynaud's within 1 year of onset of skin changes (puffy or hidebound)
2. Truncal and acral skin involvement
3. Presence of tendon friction rubs
4. Early and significance incidence of interstitial lung disease, oliguric renal failure, diffuse gastrointestinal disease, and myocardial involvement
5. Absence of anticentromere antibodies (ACA)
6. Nailfold capillary dilatation and capillary destruction
7. Antitopoisomerase antibodies (30% of patients)

Limited cutaneous SSc (lcSSc)
1. Raynaud's for years (occasionally decades)
2. Skin involvement limited to hands, face, feet, and forearms (acral) or absent
3. A significant late incidence of pulmonary hypertension, with or without interstitial lung disease, trigeminal neuralgia
4. A high incidence of anticentromere antibodies (70～80%)
5. Dilated nailfold capillary loops, usually without capillary dropout

1. 皮膚硬化

①皮膚硬化出現6年以内のdcSSc早期例，②急速な皮膚硬化の進行が認められる例，③触診で浮腫硬化が主体である例，では20～30 mg/日の少量のステロイド内服が有効であることが知られている[5]．皮膚硬化には有効でありQOL向上には役立つと考えられるが，肺線維症には無効であり生存率を向上させるかは不明である．二重盲検試験が必要であろう．しかしながら，欧米では大量ステロイド内服は強皮症腎を誘発することより使用には慎重である．

2. 末梢血管障害

SScにおける末梢血管障害に対する薬物治療は一般的にはビタミンE製剤（ユベラニコチネート®など），カルシウム拮抗薬（ヘルベッサー®など），プロスタグランジン製剤（プロサイリン®など），セロトニン拮抗薬（アンプラーグ®など），血小板凝集抑制薬（ペルサンチン®）などが頻用される．経口エンドセリン受容体拮抗薬（ボセンタン）もその有効性が知られていたが，現在保険収載（肺高血圧症に対して）となり使用可能である．四肢に潰瘍，壊疽が生じた際にはリポPGE1（リプル®など）静注や抗トロンビン薬であるアルガトロバン（ノバスタン®など）点滴が有効である．皮膚潰瘍に対する外用剤はbFGF（フィブラスト®スプレー），プロスタンジン軟膏，ユーパスタ®軟膏，アクトシン®軟膏などを用いて適切な処置を行う．

3. 消化器症状

食道症状の強い例では，節食障害，消化管出血による貧血，悪心，嘔吐を伴い，著しくQOLが低下する場合もあり，これらの症状を改善することはQOLの向上をもたらし，臨床上非常に重要である．SScの逆流性食道炎に対してH_2ブロッカーは効果が不十分であるが，プロトンポンプ阻害剤によって劇的に改善される[6]．また，エカベトナトリウム（ガストローム®）の併用においてさらに症状の改善が期待される[7]．食道蠕動運動の低下による胸のつかえ感や下部消化管蠕動運動の低下による便秘に対しては消化管蠕動運動を亢進させるクエン酸モサプリド（ガスモチン®）が有効である[8]．

下部消化管病変に対しては，消化管機能改善薬（エリスロマイシン，オクトレオチド），抗菌薬カナマイシンなどが使用される．偽性腸閉塞，嚢腫様腸管気腫には禁飲食で保存的に対応する．禁飲食で保存的に対応して改善しない場合，あるいは低栄養状態でQOLが著しく障害される場合は在宅中心静脈栄養も考慮する．

4. 肺病変

対象となる症例が少数であり，またどのような症例で有効であるのかいまだ意見の一致をみないが（著者の経験では早期例というよりはむしろ蜂巣肺を呈する進行例で有効と考えられるが），シ

クロフォスファミド静注あるいは経口投与の有効性が報告されている[9]．肺高血圧症に対しては，予防としてはプロスタグランジン製剤内服があげられる．経口エンドセリン受容体拮抗薬（ボセンタン）も効果はいまだ不十分といえよう．プロスタサイクリン持続静注療法，シルデナフィル，タダラフィル，アンブリセンタンの有効性も報告されているが，どの薬剤も単剤では効果不十分といえよう．

5．腎 病 変

強皮症腎クリーゼに対しては，アンギオテンシン変換酵素阻害剤で短期作用型のカプトリルが第1選択となる．ただちにカプトリルを開始し，漸次増量して血圧の正常化を目指す．血圧調節困難あるいは副作用などで継続困難である場合，カルシウム拮抗薬あるいはアンギオテンシンII受容体阻害薬を併用ないし変更する．腎機能悪化が進行する場合降圧療法継続とともに早期に血液透析療法の導入を考慮する．

6．心 病 変

心嚢液貯留に対して中等量ステロイドないし利尿薬，心筋障害に対してはβブロッカー，Ca拮抗薬，不整脈に対しては抗不整脈薬およびペースメーカーにおいて治療を行うことが推奨されている．

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全身性強皮症

尹　浩信*

全身性強皮症(systemic sclerosis：SSc)は皮膚の線維性変化を主徴とし，ときに内蔵諸臓器の線維化を伴う．線維化の病態形成は，血管内皮細胞傷害，リンパ球や単球の活性化にはじまり，線維芽細胞における細胞外マトリックス調節異常に至ると考えられており，線維芽細胞の活性化が線維化の過程で重要な役割を果たしていると考えられている．SScは均一な疾患ではなく，多様な臨床像を呈するため，その診断，評価には各種診断基準，病型分類，特異抗核抗体が用いられ，また皮膚病変，内臓病変の精査の後，個々の患者の重症度・予後を判定し，治療方針を決定することが重要である．本稿ではSScの診断および治療方針の立て方についてわが国の治療指針を用いて概説した．

はじめに

全身性強皮症(systemic sclerosis：SSc)は均一な疾患ではなく，多様な臨床像を呈するため，その診断，評価には各種診断基準，病型分類，特異抗核抗体が用いられ，また皮膚病変，内臓病変の精査の後，個々の患者の重症度・予後を判定し，治療方針を決定することが可能となる．本稿ではSScの診断および治療方針の立て方についてわが国の治療指針を用いて概説した．

1．SScの分類基準

SScの診断に際しては国際的にはアメリカリウマチ協会の分類予備基準が広く参考とされていた(**表1**)[1]．大基準である近位皮膚硬化が存在するか，あるいは小基準(強指症，指尖陥凹状瘢痕，両側性肺線維症)の2項目以上が存在すればSScと診断される．

2003年に厚生労働省強皮症調査研究班(班長：竹原和彦教授)によって，わが国におけるSSc診断基準(**表2**)が改定された[2]．以前の研究班による診断基準は項目数が多く，記憶しにくいという問題点があったが，今回の改定では国際的に広く用いられていたアメリカリウマチ協会の分類予備基準をそのまま採用し，小基準を1項目追加した形となり，記憶しやすくなった．加えられた小項目は「抗トポイソメラーゼI(Scl-70)抗体または抗セントロメア抗体陽性」であり，これらの自己抗体は，SSc特異的でSScの診断上不可欠であり，また保険収載となっており一般臨床の場でも容易に測定可能である．

また2013年に改定された米国リウマチ学会(ACR)と欧州リウマチ学会(EULAR)による分類基準も用いられるようになっている(**表3**)[3]．本基準は早期例の診断にも有用となるように改訂されている．この欧米の診断基準の改定を受け，平成26年度厚生労働科学研究費補助金難治性疾患

〔キーワード〕
skin sclerosis
vascular damage
antinuclear antibodies
pulmonary fibrosis

*IHN Hironobu/熊本大学大学院生命科学研究部皮膚病態治療再建学分野

表 1. 全身性強皮症の分類予備基準
(アメリカリウマチ協会, 1980[1]より引用)

大基準	近位皮膚硬化 (手指あるいは足趾より近位に及ぶ皮膚硬化)
小基準	1．手指あるいは足趾に限局する皮膚硬化 2．指尖陥凹性瘢痕，あるいは指腹の萎縮 3．両側性の肺基底部の肺線維症

大基準あるいは小基準2項目以上を満足すればSScと診断
ただし限局性強皮症と *pseudoscleroderma tous disorder* を除外する

表 2. 厚生労働省強皮症研究班による全身性強皮症の診断基準(佐藤伸一, 2004[2]より引用)

大基準
手指あるいは足趾を越える皮膚硬化*

小基準
① 手指あるいは足趾を限局する皮膚硬化
② 手指尖端の陥凹性瘢痕，あるいは指腹の萎縮**
③ 両側性肺基底部の線維症
④ 抗トポイソメラーゼⅠ(Scl-70)抗体または抗セントロメア抗体陽性

判定：大基準あるいは小基準①および②～④の1項目以上を満たせば全身性強皮症と診断

*限局性強皮症(いわゆるモルフィア)を除外する
**手指の循環障害によるもので，外傷などによるものを除く

等政策研究事業研究課題名「強皮症・皮膚線維化疾患の診断基準・重症度分類・診療ガイドライン作成事業」(研究代表者　尹　浩信)[4]にて全身性強皮症の診断基準・重症度分類・治療指針が現在改定中である．

2．皮膚硬化と病型分類

SScにおいては皮膚硬化が最も重要な症状である．皮膚硬化の有無，程度は前腕伸側の皮膚生検による病理組織像で確認することが必要であり，指定難病の申請にも記載が求められている．

皮膚硬化の範囲により病型分類がなされ，現在ではLeRoyの分類が一般的に用いられている．LeRoyら[5]は限局皮膚硬化型全身性強皮症(limited cutaneous systemic sclerosis：lcSSc)とびまん皮膚硬化型全身性強皮症(diffuse cutaneous systemic sclerosis：dcSSc)の2型に分類することを提唱し，世界的に広く使用されている．この分類は皮膚硬化の範囲だけではなく，いくつかの臨床所見，検査所見を加味した総合的分類である(**表4**)．

またSScでは抗核抗体の陽性率が95%以上と高く，さまざまな核抗原に対する自己抗体が検出され，抗核抗体の検出はSScの診断や病型分類に有用である．SSc疾患特異性の高い，抗トポイソメラーゼⅠ抗体はわが国SScの約30%で陽性となり，dcSScに多く，間質性肺疾患(interstitial lung disease：ILD)，皮膚潰瘍と相関する．抗セントロメア抗体はわが国SScの約30%で陽性となり，lcSScに多く，内臓病変の合併は少ないが10%程度に肺動脈性肺高血圧症(pulmonary arterial hypertension：PAH)を合併し，ときに原発性胆汁性肝硬変(primary biliary cirrhosis：PBC)を合併することが知られている．なお抗セントロメア抗体はときにほかの膠原病でも陽性となることに留意を要する．抗RNAポリメラーゼⅢ抗体はわが国SScの数%で陽性となり，dcSScに多く，腎クリーゼと相関する．抗U1RNP抗体はわが国SScの10～15%で陽性となり，ILD，色素沈着と相関があり，overlap症候群が多いことが知られている．なお抗U1RNP抗体は全身性エリテマトーデス(systemic lupus erythematosus：SLE)や混合性結合組織病(mixed connective tissue disease：MCTD)でも検出されるので注意を要する．

3．SScの治療指針

現在SScの基礎治療薬として症状全般に有効であると考えられる薬剤などはなく，皮膚を含め

表 3. 強皮症分類基準 2013(van den Hoogen F *et al*, 2013[3]より引用)

大基準	
近位皮膚硬化(両側 MP 関節を越える皮膚硬化)	9 点
小基準	
① 強指症：指の腫脹のみ	2 点
PIP 関節と MP 関節間の皮膚硬化	4 点
② 手指尖端の病変：PIP 関節より遠位の潰瘍	2 点
陥凹性瘢痕	3 点
③ 毛細血管拡張	2 点
④ 爪上皮毛細血管異常(≒爪上皮出血点)	2 点
⑤ 肺高血圧症/間質性肺炎	2 点
⑥ レイノー現象	3 点
⑦ 抗セントロメア抗体/抗トポイソメラーゼⅠ抗体/抗 RNA ポリメラーゼⅢ抗体	3 点

9 点以上で SSc と診断
①～⑦ の項目は各項目点数の高い 1 つのみ算定する
強指症がない症例は適応外，NSF，generalized morphea，好酸球性筋膜炎，硬化性粘液水腫
糖尿病性浮腫性硬化症，肢端紅痛症，ポルフィリン症，硬化性苔癬，GVHD，手関節症

た各臓器の重症度を把握し，症例毎，臓器毎に治療を考慮することが重要である．2004 年に厚生労働省強皮症調査研究班が作成した「全身性強皮症における重症度分類・治療指針試案(2004)」(http://www. nanbyou. or. jp/pdf/019_1. pdf#search＝'強皮症＋重症度分類')と 2010 年に厚生労働省強皮症調査研究班が作成した「全身性強皮症強皮症診療ガイドライン(2010)」(http://derma.w3.kanazawa-u.ac.jp/SSc/pamphret/pdf/guidelines.pdf#search＝'全身性強皮症強皮症診療ガイドライン')が参考となるので是非一読をお勧めする．以下に臓器毎の治療指針を簡単に記す．

1）皮膚硬化

① 皮膚硬化出現 6 年以内の dcSSc 早期例，② 急速な皮膚硬化の進行が認められる例，③ 触診にて浮腫硬化が主体である例，では 20～30 mg/日の少量～中等量のステロイド内服が有効であることが知られている[6]．皮膚硬化には有効であり QOL 向上には役立つと考えられるが，肺線維症には無効であり生存率を向上させるかは不明である．二重盲検試験が必要であろう．しかしながら，欧米では大量ステロイド内服は強皮症腎を誘発することより使用には慎重である．

2）末梢血管障害

SSc における末梢血管障害に対する薬物治療は一般的にはビタミン E 製剤(トコフェロールニコチン酸エステルなど)，カルシウム拮抗薬(ジルチアゼム塩酸塩など)，プロスタグランジン製剤(ベラプロストナトリウムなど)，セロトニン拮抗薬(サルポグレラート塩酸塩など)，血小板凝集抑制薬(ジピリダモール)などが頻用される．経口エンドセリン受容体拮抗薬(ボセンタン水和物)もその有効性が知られていいたが，現在保険収載(肺高血圧症に対して)となり使用可能である．四肢に潰瘍，壊疽が生じた際にはリポ PGE1(アルプロスタジルなど)静注や抗トロンビン薬であるアルガトロバン点滴が有効である．皮膚潰瘍に対する外用剤は bFGF(トラフェルミン)，アルプロスタジルアルファデクス軟膏，白糖・ポビドンヨード配合軟膏，ブクラデシンナトリウム軟膏などを用い

表 4. LeRoy による全身性強皮症病型分類(LeRoy EC *et al*, 1988[5]より引用)

diffuse cutaneous SSc (dcSSc)

1. Onset of Raynaud's within 1 year of onset of skin changes (puffy or hidebound)
2. Truncal and acral skin involvement
3. Presence of tendon friction rubs
4. Early and significant incidence of interstitial lung disease, oliguric renal failure, diffuse gastrointestinal disease, and myocardial involvement
5. Absence of anticentromere antibodies (ACA)
6. Nailfold capillary dilatation and capillary destruction
7. Antitopoisomerase antibodies (30% of patients)

Limited cutaneous SSc (lcSSc)

1. Raynaud's for years (occasionally decades)
2. Skin involvement limited to hands, face, feet, and forearms (acral) or absent
3. A significant late incidence of pulmonary hypertension, with or without interstitial lung disease, trigeminal neuralgia
4. A high incidence of anticentromere antibodies (70～80%)
5. Dilated nailfold capillary loops, usually without capillary dropout

て適切な処置をおこなう.

3）消化器症状

食道症状の強い例では，節食障害，消化管出血による貧血，悪心，嘔吐を伴い，著しくQOLが低下する場合もあり，これらの症状を改善することはQOLの向上をもたらし，臨床上非常に重要である．SScの逆流性食道炎に対してH_2ブロッカーは効果が不十分であるが，プロトンポンプ阻害薬によって劇的に改善される[7]．またエカベトナトリウム水和物の併用にてさらに症状の改善が期待される[8]．食道蠕動運動の低下による胸のつかえ感や下部消化管蠕動運動の低下による便秘に対しては消化管蠕動運動を亢進させるモサプリドクエン酸塩水和物が有効である[9]．

下部消化管病変に対しては，消化管機能改善薬(エリスロマイシン，オクトレオチド酢酸塩)，抗菌薬カナマイシン硫酸塩などが使用される．偽性腸閉塞，嚢腫様腸管気腫には禁飲食で保存的に対応する．禁飲食で保存的に対応して改善しない場合，あるいは低栄養状態でQOLが著しく障害される場合は在宅中心静脈栄養も考慮する．

4）肺病変

対象となる症例が少数であり，またどのような症例で有効であるのかいまだ意見の一致を見ないが〔筆者の経験では早期例というよりはむしろ蜂巣肺を呈する進行例で有効と考えられるが(%VC 70%未満，高解像度CTにて肺野の20%以上に病変が及ぶ場合)〕，シクロホスファミド水和物静注あるいは経口投与の有効性が報告されている[10]．肺高血圧症に対しては，予防としてはプロスタグランジン製剤内服があげられる．経口エンドセリン受容体拮抗薬(ボセンタン水和物)も効果はいまだ不十分と言えよう．プロスタサイクリン持続静注療法，シルデナフィルクエン酸塩，タダラフィル，アンブリセンタンの有効性も報告されているが，どの薬剤も単剤では効果不十分と言えよう．

5）腎病変

強皮症腎クリーゼに対しては，アンジオテンシン変換酵素阻害薬で短時間作用型のカプトプリルが第一選択となる．直ちにカプトプリルを開始し，漸次増量して血圧の正常化を目指す．血圧調節困難あるいは副作用などで継続困難である場合，カルシウム拮抗薬あるいはアンジオテンシンⅡ受容体阻害薬を併用ないし変更する．腎機能悪化が進行する場合降圧療法継続とともに早期に血液透析療法の導入を考慮する．

6）心病変

心嚢液貯留に対して中等量ステロイドないし利尿薬，心筋障害に対してはβ遮断薬，Ca 拮抗薬，不整脈に対しては抗不整脈およびペースメーカーにて治療をおこなうことが推奨されている．

おわりに

前述したように「強皮症・皮膚線維化疾患の診断基準・重症度分類・診療ガイドライン作成事業」にて SSc の診断基準・重症度分類・治療指針が改定され，今後報告される予定である．公表後は是非一読をお勧めする．

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Multifaceted contribution of the TLR4-activated IRF5 transcription factor in systemic sclerosis

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Systemic sclerosis (SSc) is a multisystem autoimmune disorder with clinical manifestations resulting from tissue fibrosis and extensive vasculopathy. A potential disease susceptibility gene for SSc is IFN regulatory factor 5 (*IRF5*), whose SNP is associated with milder clinical manifestations; however, the underlying mechanisms of this association remain elusive. In this study we examined IRF5-deficient (*Irf5*$^{-/-}$) mice in the bleomycin-treated SSc murine model. We show that dermal and pulmonary fibrosis induced by bleomycin is attenuated in *Irf5*$^{-/-}$ mice. Interestingly, we find that multiple SSc-associated events, such as fibroblast activation, inflammatory cell infiltration, endothelial-to-mesenchymal transition, vascular destabilization, Th2/Th17 skewed immune polarization, and B-cell activation, are suppressed in these mice. We further provide evidence that IRF5, activated by Toll-like receptor 4 (TLR4), binds to the promoters of various key genes involved in SSc disease pathology. These observations are congruent with the high level of expression of IRF5, TLR4, and potential endogenous TLR4 ligands in SSc skin lesions. Our study sheds light on the TLR4-IRF5 pathway in the pathology of SSc with clinical implications of targeting the IRF5 pathways in the suppression of disease development.

interferon regulatory factor 5 | systemic sclerosis | fibrosis | vasculopathy | Toll-like receptor 4

Systemic sclerosis (SSc) is a multisystem connective tissue disease characterized by immune abnormalities, vasculopathy, and extensive tissue fibrosis (1). Based on the results of etiological and genetic studies, the conventional wisdom is that SSc is caused by a complex interplay between genetic factors and environmental influences. For instance, the biggest risk factor for SSc is family history (2). On the other hand, concordance for SSc is around 5% in twins and is similar in monozygotic and dizygotic twins, whereas antinuclear antibodies are detected more frequently in the healthy monozygotic twin sibling than in the healthy dizygotic twin sibling of an SSc patient (3). In addition, most SSc susceptibility genes are HLA haplotypes and non-HLA immune-related genes that are shared by other collagen diseases (4). Therefore, genetic factors are likely associated with autoimmunity, increasing the susceptibility to autoimmune diseases including SSc, but additional environmental factors are required to induce clinically definite SSc in genetically predisposed individuals. Despite these etiological and genetic data, the entire process of the SSc development and pathogenesis remains elusive.

Therefore it is important to elucidate the molecular mechanism(s) underlying SSc pathogenesis. In this regard, much attention has been focused recently on the innate immune signaling via Toll-like receptors (TLRs) in various pathological conditions. For instance, fibroblasts and endothelial cells in SSc lesional skin highly express TLR4, originally identified as the receptor for bacterial LPS, and TLR4 signaling amplifies the sensitivity to TGF-β in dermal fibroblasts (5–7). It also was shown that dermal and lung fibrosis is attenuated in bleomycin (BLM)-treated TLR4-deficient mice (7). Endogenous potential TLR4 ligands are up-regulated in SSc lesional skin (5–7), and serum levels correlate with severe organ involvement and immunological abnormalities (8, 9). Therefore, the TLR4 signaling pathway is suspected to play a central role in the SSc pathogenesis.

Although how the TLR4 signaling pathway contributes to SSc pathogenesis remains enigmatic, it is interesting that several independent case-control and genome-wide association studies identify IFN regulatory factor 5 (*IRF5*), a member of the IFN regulatory factor (IRF) family, as an SSc susceptibility gene (10–15). IRFs were identified primarily in the research of the type I IFN system and have been shown to have functionally diverse roles in the regulation of the innate and adaptive immune responses (16). Reflecting such property of IRFs, SNPs of IRFs have been linked to the development of various immune and inflammatory disorders. IRF5 is of particular interest, being implicated in multiple sclerosis, systemic lupus erythematosus, rheumatoid arthritis, and SSc (14). Thus far an association of certain SNPs within the *IRF5* promoter with the risk and severity of SSc has been reported (10–15), but whether and how IRF5 is activated to contribute to disease development remains unknown.

Stimulation of TLRs triggers the activation of myeloid differentiation factor 88 (MyD88)-dependent and/or independent pathways (16). IRF5 is activated via the MyD88 pathway in dendritic cells and macrophages (17). TLR-activated IRF5 mediates the induction of genes IL-6, IL-12, and TNF-α (17). Hence, an intriguing possibility is that TLR4-mediated activation

Significance

The present study is, to our knowledge, the first demonstration of the molecular mechanisms underlying the association of IFN regulatory factor 5 (IRF5) expression with milder clinical manifestations of systemic sclerosis (SSc). It is speculated that endogenous ligands induce Toll-like receptor 4 signaling and promote IRF5 transcriptional regulation of its target gene promoters, which may be required for the development of SSc. Our present study supports this notion. Symptoms associated with SSc in humans were suppressed in mice deficient in the *Irf5* gene. As such, this study offers previously unidentified insight into the complexity of SSc pathology, giving impetus to further clinical studies for the treatment of the disease.

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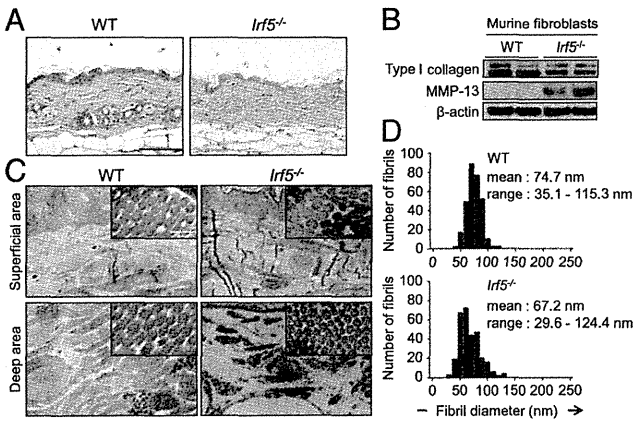

Fig. 1. Loss of *Irf5* impaired collagen metabolism and fibrillogenesis in vivo. (*A*) Representative skin image of WT and *Irf5*$^{-/-}$ mice. (Scale bar, 100 μm.) (*B*) Immunoblotting with cell lysates from WT and *Irf5*$^{-/-}$ murine dermal fibroblasts. (*C*) Ultrastructure of dermal collagen fibrils evaluated by electron microscopy. (Yellow scale bar, 200 nm; red scale bar, 2 μm.) (*D*) Frequency distribution profiles of fibril diameter in the dermis of WT and *Irf5*$^{-/-}$ mice (500 collagen fibrils per group).

of IRF5 is involved in SSc. We therefore studied the role of IRF5 in the regulation of genes associated with the susceptibility to and the severity of SSc using IRF5-deficient mice in the context of TLR4 signaling. We show that IRF5, activated by TLR4, binds to the promoters of various key genes involved in the disease symptoms. We discuss our findings in terms of the complexity of SSc and its clinical implications.

Results

Involvement of IRF5 in the Fibrosis- and Fibrillogenesis-Related Genes in Dermal Fibroblasts. First, to investigate the role of IRF5 in skin homeostasis, we examined by histology the skin of *Irf5*$^{-/-}$ mice (12 wk after birth) without BLM treatment. As shown in Fig. 1*A*, thinner collagen bundles were found in the dermis of *Irf5*$^{-/-}$ mice than in the dermis of WT littermate mice, but other skin structures in *Irf5*$^{-/-}$ mice looked normal. Consistent with this finding, collagen content decreased in the skin of *Irf5*$^{-/-}$ mice (Fig. S1*A*), and *Irf5*$^{-/-}$ dermal fibroblasts exhibited lower expression of type I collagen and higher expression of matrix metallopeptidase (MMP)-13 (Fig. 1*B* and Fig. S1*B*). Further analyses with electron microscopy delineated thinner collagen fibrils, especially in deep dermis (Fig. 1*C*), and higher variability in fibril diameter in *Irf5*$^{-/-}$ mice (Fig. 1*D*), suggesting an abnormality in collagen fibrillogenesis.

Interestingly, the ChIP assay revealed IRF5 binding to the promoters of the collagen, type 1, α1 (*Col1a1*), collagen, type 1, α2 (*Col1a2*), and *Mmp13* genes, indicating the potential involvement of IRF5 in the regulation of these genes (Fig. S1*C*). In addition, a notable decrease in the mRNAs for fibrillogenesis-related genes, namely a disintegrin and metalloprotease domain with thrombospondin type 1, motif 2 (*Adamts2*), lysyl oxidase (*Lox*), decorin (*Dcn*), and lumican (*Lum*), was observed in *Irf5*$^{-/-}$ dermal fibroblasts as compared with WT dermal fibroblasts (Fig. S1*D*). A similar mRNA expression pattern also was detected in the skin of *Irf5*$^{-/-}$ mice (Fig. S1*E*). Thus, IRF5, perhaps constantly but weakly activated in these mice, may influence extracellular matrix homeostasis by regulating fibrosis- and fibrillogenesis-associated gene expression in dermal fibroblasts (see below). It is worth noting that, except for the *Dcn* gene, these gene-expression profiles are contrary to those of SSc (18).

TLR4-Activated IRF5 Regulates *COL1A2* Gene Expression in Dermal Fibroblasts. In addition to murine dermal fibroblasts, we also detected IRF5 binding to the promoters for the *COL1A1*, *COL1A2*, and *MMP1* genes in human dermal fibroblasts (Fig. 2*A*). In the case of the *COL1A2* promoter, sequence-specific binding of IRF5 to the IFN-stimulated response element (ISRE) was confirmed by an oligonucleotide pull-down assay (Fig. 2*B*). We further analyzed the role of IRF5 in *COL1A2* gene expression in human dermal fibroblasts by a transient assay using a *COL1A2*-promoter-Luciferase gene and found that ectopic expression of IRF5 increased *COL1A2* promoter activity in a dose-dependent manner (Fig. 2*C*). Of note, LPS and TGF-β, known to induce the expression of type I collagen (5), further augmented the reporter gene expression (Fig. 2*C*), suggesting that TLR4 signaling regulates the transcriptional activity of IRF5. Indeed, IRF5 binding to the *COL1A2* promoter was enhanced significantly by simultaneous stimulation of LPS and TGF-β1 (Fig. 2*D*). Interestingly, the enhancement of IRF5 binding to the *COL1A2* promoter also was observed when the cells were stimulated by high-mobility group box 1 (HMGB1), which is also known to activate TLR4 in lieu of LPS (Fig. 2*E*) (19). Consistent with this notion, ChIP in *Tlr4*$^{-/-}$ murine dermal fibroblasts showed a remarkable decrease in IRF5 binding to the *Col1a2* promoter (Fig. 2*F*), but *Tlr4* deficiency did not affect the expression of IRF5 (Fig. 2*G*). Thus, these observations underscore the evidence that the TLR4–IRF5 axis induces *COL1A2* gene expression in dermal fibroblasts.

Attenuated Dermal and Pulmonary Fibrosis in BLM-Treated *Irf5*$^{-/-}$ Mice. The role of IRF5 in tissue fibrosis was investigated further in BLM-treated mice. In line with in vitro data, dermal thickness, collagen content, and the number of myofibroblasts were significantly decreased in BLM-treated *Irf5*$^{-/-}$ mice as compared with BLM-treated WT mice (Fig. 3*A* and Fig. S2 *A*–*C*). The expression profile of fibrosis- and fibrillogenesis-related genes in *Irf5*$^{-/-}$ dermal fibroblasts, which was confirmed at the protein level by immunostaining for MMP-13 (Fig. S2 *E* and *F*), was maintained in the skin of BLM-treated *Irf5*$^{-/-}$ mice (Fig. S2*D*). Also, lung histology revealed less fibrosis, less destruction of

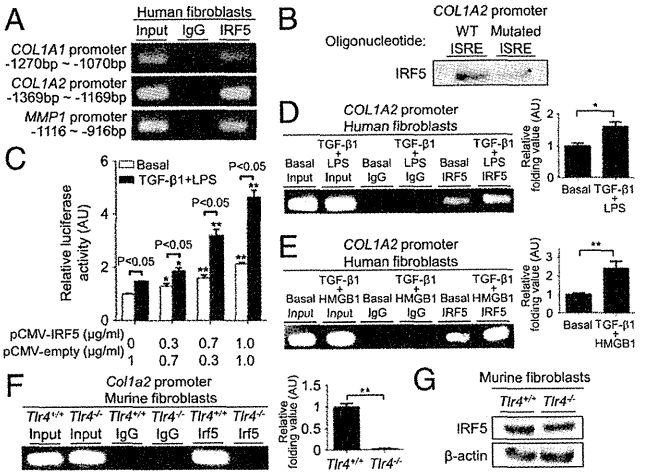

Fig. 2. TLR4-activated IRF5 induces the profibrotic phenotype in dermal fibroblasts. (*A*, *D*, and *E*) ChIP analysis with anti-IRF5 antibody in human dermal fibroblasts ($n = 4$). (*B*) Proteins pulled down by oligonucleotides including WT or mutated ISRE of the *COL1A2* promoter were subjected to immunoblotting with anti-IRF5 antibody. (*C*) Luciferase assay with the *COL1A2* promoter construct in human dermal fibroblasts ($n = 4$). Significant differences shown with asterisks are compared with the columns of the same color at the far left. (*F* and *G*) ChIP assay (*F*) and immunoblotting (*G*) with anti-IRF5 antibody in *Tlr4*$^{+/+}$ and *Tlr4*$^{-/-}$ murine dermal fibroblasts. In *C*–*E*, some cells were stimulated with TGF-β1 and LPS or HMGB1 for 24 h. In *D*–*F*, quantification by qRT-PCR is shown in the right panels ($n = 5$). $^{*}P < 0.05$ and $^{**}P < 0.01$ by two-tailed unpaired *t*-test. AU, arbitrary units.

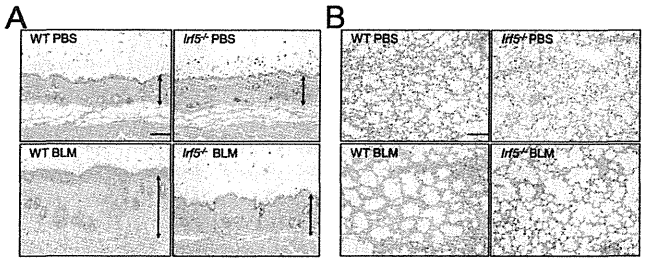

Fig. 3. Deletion of *Irf5* attenuates BLM-induced dermal and pulmonary fibrosis. Representative sections of skin (*A*) and lung (*B*) in WT and *Irf5*$^{-/-}$ mice injected with PBS or BLM. Vertical bars with arrows represent dermal thickness. (Horizontal scale bars, 100 μm.)

alveolar structures, and less inflammatory cell infiltration in BLM-treated *Irf5*$^{-/-}$ mice than in BLM-treated WT mice (Fig. 3*B* and Fig. S2*G*). Thus, *Irf5* deficiency suppresses pathological dermal and pulmonary fibrosis in BLM-treated mice.

Promotion of Th1 Immune Polarization and Attenuation of B-Cell Activation by the Absence of IRF5. We next asked whether the loss of IRF5 also influences immune cells in BLM-treated mice. As shown in Fig. 4*A*, immunostaining analysis revealed reduced infiltration of T cells, B cells, mast cells, and macrophages in the skin and lungs of BLM-treated *Irf5*$^{-/-}$ mice (see also Fig. S3*A*), suggesting that IRF5 is involved in the BLM-induced inflammatory responses.

Consistent with this observation, the expression of mRNAs for fibrosis-related cytokines and chemokines was different in the skin and lungs of BLM-treated WT and *Irf5*$^{-/-}$ mice; IFN-γ mRNA levels were increased significantly, but IL-4 and IL-6 mRNA levels were decreased in the absence of IRF5 (Fig. S3 *B* and *C*). These observations suggested a Th1-type immune polarization in BLM-treated *Irf5*$^{-/-}$ mice. This notion was confirmed by intracellular flow cytometry for cytokines and master transcription factors within lymphocytes of draining lymph nodes, showing an increase in Th1 cells but no change of Th2 and Th17 cells in BLM-treated *Irf5*$^{-/-}$ mice as compared with BLM-treated WT mice (Fig. 4 *B* and *C* and Fig. S3 *D* and *E*).

In this regard, we also measured by ChIP assay IRF5 binding to the T-box expressed in T cells (*Tbet*) promoter in CD4$^+$ T cells. The results suggest that the absence of IRF5 may promote Th1 polarization through induction of the T-bet transcription factor; in other words, IRF5 may serve as a repressor of the *Tbet* gene (Fig. 4*D*). IRF5 is known to function as a positive regulator of various genes in immune cells (17), but a transcriptional repressive function has not been reported. To confirm this notion, we carried out a reporter gene-based assay in which activation of a *Tbet* promoter induces the expression of luciferase (Tbet-Luc) in HEK293T cells. When IRF5A, a constitutive active type isoform of IRF5 which lacks a nuclear export signal (20), was coexpressed with Tbet-Luc, luciferase activity was suppressed in a dose-dependent manner (Fig. 4*E*), indicating the repressive action of IRF5 on this promoter. In view of a previously published report that TLR4 signaling in T cells promotes an inflammatory response (21), one may envisage that the TLR4–IRF5 axis also is involved in the BLM-induced immune pathogenesis.

We also extended our study to the role of IRF5 in B cells. We observed that expression of CD19, a critical positive-response regulator (22), was expressed at lower levels in IRF5-deficient B cells (Fig. S3*F*). Furthermore, IRF5-deficient B cells produced lower amounts of IL-6 than did WT B cells in response to LPS and/or anti-CD40 antibody (Fig. S3*G*). Of note, the *Cd19* and *Il6* promoters also were bound by IRF5 (Fig. 4*F* and Fig. S3*H*), and the sequence-specific binding of IRF5 to the ISRE of the *Il6* promoter also was confirmed (Fig. S3*I*).

It is interesting that IRF5 binding to the *Tbet* and *Il6* promoters also was suppressed in *Tlr4*$^{-/-}$ CD4$^+$ T cells and *Tlr4*$^{-/-}$ B cells, respectively (Fig. 4 *D* and *F*). Furthermore, enhanced IRF5 binding to the *Il6* promoter by HMGB1 also was observed in B cells (Fig. 4*F*). Overall, these results indicate that TLR4-activated IRF5 is directly involved in the regulation of Th1 cell differentiation and B-cell activation.

Regulation of Antifibrotic Property by IRF5 in Endothelial Cells. We next examined the vascular aspect of BLM-treated *Irf5*$^{-/-}$ mice by focusing on cell-adhesion molecules, vascular stability, and endothelial-to-mesenchymal transition (EndoMT). In the skin of BLM-treated mice, selectin P (*Selp*) and selectin E (*Sele*) mRNA levels were increased by the absence of IRF5, but intercellular adhesion molecule 1 (*Icam1*) mRNA levels were decreased (Fig. S4*A*). In the lung, the lack of IRF5 significantly reduced *Selp* and *Icam1* and especially glycosylation-dependent cell-adhesion molecule 1 (*Glycam1*) mRNA expression levels (Fig. S4*B*). Given that L-selectin, a ligand for GlyCAM-1, and ICAM-1 promote Th2 and Th17 cell infiltration, whereas P-selectin and

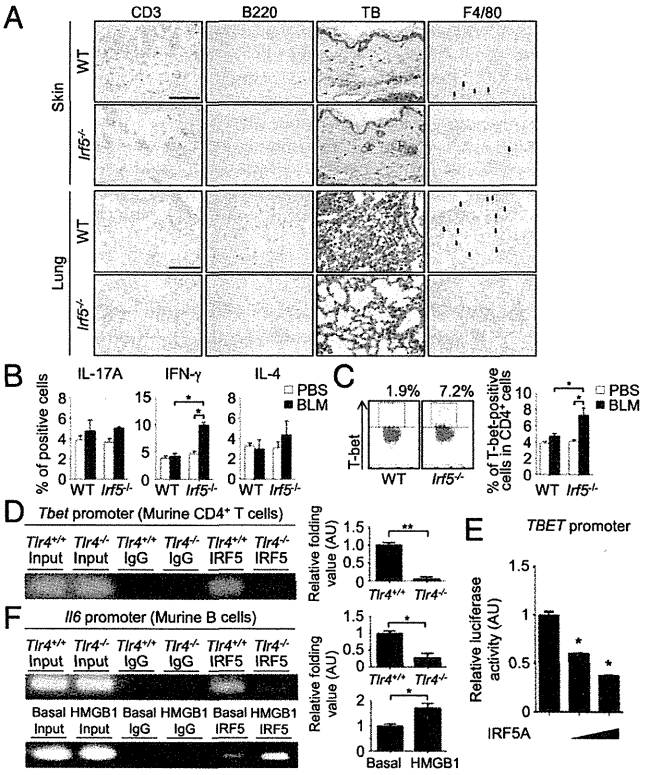

Fig. 4. Loss of *Irf5* suppresses the induction of profibrotic inflammatory responses in BLM-treated mice. (*A*) Immunohistochemistry for CD3, B220, and F4/80 and toluidine blue (TB) staining in the skin and lung of BLM-treated WT and *Irf5*$^{-/-}$ mice. (Scale bars, 100 μm.) Arrows indicate F4/80$^+$ cells ($n = 5$). (*B* and *C*) Percentage of IL-17A–, IFN-γ–, and IL-4–producing CD4$^+$ T cells (*B*) and T-bet$^+$ CD4$^+$ T cells (*C*) in draining lymph nodes of BLM-treated mice determined by intracellular staining ($n = 4$). Representative FACS plots of intracellular T-bet staining are shown in *C*. (*D* and *F*) ChIP assay with anti-IRF5 antibody in murine CD4$^+$ T cells (*D*) and B cells (*F*) with or without homozygous *Tlr4* deletion. In some experiments, B cells were stimulated with 10 μg/mL of HMGB1 for 24 h. For each ChIP assay, quantification by qRT-PCR is shown in the panels at right ($n = 5$). (*E*) Luciferase assay using the *TBET* promoter constructs in HEK293T cells cotransfected with IRF5A expression vector ($n = 4$). $^*P < 0.05$ and $^{**}P < 0.01$ by two-tailed unpaired *t*-test.

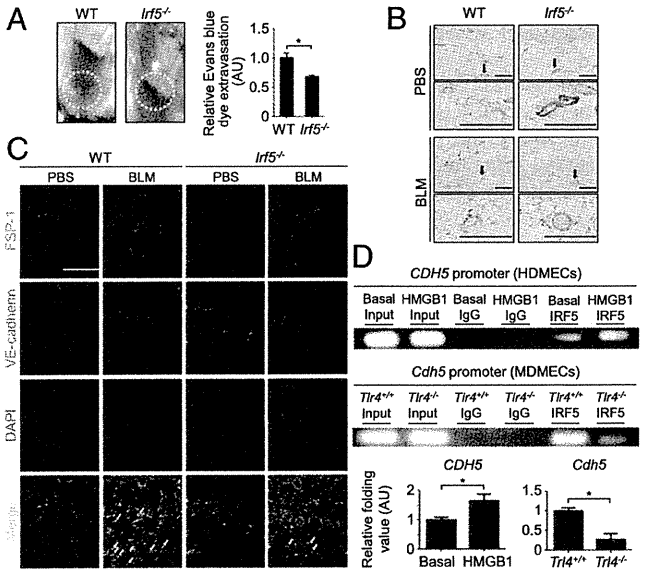

Fig. 5. *Irf5* deletion abrogates vascular destabilization and EndoMT induced by BLM. (*A, Left*) Evaluation for the extravasation of Evans blue dye injected into the caudal vein of BLM-treated mice. (*Right*) Quantification of Evans blue dye extravasation by formamide extraction (n = 6). (*B*) Immunohistochemistry for α-SMA in the skin of PBS- or BLM-treated mice. (Scale bars, 100 μm.) (*C*) Immunofluorescence staining for FSP1 (green), VE-cadherin (red), and DAPI (blue) in skin samples from each group. Arrows represent FSP1/VE-cadherin double-positive cells. (Scale bar, 100 μm.) (n = 4). (*D, Upper*) ChIP assay with anti-IRF5 antibody in human dermal microvascular endothelial cells with or without HMGB1 stimulation (10 μg/mL for 24 h) and in $Tlr4^{+/+}$ and $Tlr4^{-/-}$ murine dermal microvascular endothelial cells. (*Lower*) Quantification by qRT-PCR (n = 5). *P < 0.05 by two-tailed unpaired t-test.

E-selectin facilitate Th1 cell infiltration, IRF5 may modulate the expression of cell-adhesion molecules, resulting in the promotion of Th1 cell infiltration in BLM-treated mice; this activity is consistent with T-helper cytokine profiles in the skin and lungs of BLM-treated mice (Fig. S3 *B* and *C*).

In the vascular permeability assay, the absence of IRF5 attenuated Evans blue dye extravasation induced by BLM (Fig. 5*A*), suggesting that IRF5 has a suppressive role in vascular stabilization. Because a mural cell phenotype contributes to vascular maturation, and α-smooth muscle actin (α-SMA) serves as a marker of mature vessels (23), α-SMA expression in dermal small vessels was evaluated by immunohistochemistry. The loss of IRF5 augments α-SMA expression, irrespective of injected agents (Fig. 5*B*). In agreement with this observation, dermal microvascular endothelial cells from $Irf5^{-/-}$ mice expressed PDGF-B and vascular endothelial (VE)-cadherin, which stabilize vasculature by acting on mural cells, at higher levels than cells from WT counterparts (Fig. S4*C*). Furthermore, IRF5 also bound the *Pdgfb* and cadherin-5 (*Cdh5*) promoters as measured by ChIP assay (Fig. S4*D*). Collectively, *Irf5* deficiency is likely to promote vascular stabilization by directly inducing the expression of PDGF-B and VE-cadherin.

Another critical event in the pathology of tissue fibrosis, EndoMT, was assessed further by double immunofluorescence for fibroblast secretory protein-1 (FSP1), a fibroblast marker, and VE-cadherin, an endothelial cell marker, revealing that *Irf5* deletion suppressed BLM-dependent induction of double-positive cells (Fig. 5*C* and Fig. S4*E*). Importantly, in human dermal microvascular endothelial cells (HDMECs), IRF5 was bound to the promoter of the snail family zinc finger 1 (*SNAIL1*) gene, encoding an important regulator of EndoMT (Fig. S4*F*). Therefore, in BLM-treated mice *Irf5* deficiency appears to inhibit EndoMT directly, contributing to the attenuation of tissue fibrosis.

In HDMECs, IRF5 was bound to the promoters of various genes, including the *ICAM1*, *PDGFB*, *CDH5*, and *SNAIL1* genes (Fig. S4*F*). When HDMECs were treated with HMGB1, the occupancy of the *CDH5* promoter by IRF5 was increased (Fig. 5*D*). Furthermore, $Tlr4^{-/-}$ murine dermal microvascular endothelial cells exhibited a marked reduction of IRF5 binding to the *Cdh5* promoter (Fig. 5*D*). These data indicate that IRF5 regulates the profibrotic/antifibrotic and proangiogenic/angiostatic phenotypes of endothelial cells, depending on TLR4 activation status. Overall, our results in toto suggest a multifaceted transcriptional regulation by IRF5, functioning as either a transcriptional activator or a repressor in this SSc model.

IRF5 Expression and Its Clinical Correlation in SSc Patients. Finally, we evaluated the association of IRF5 expression with clinical features in SSc patients. *IRF5* mRNA expression was elevated significantly in SSc lesional skin compared with healthy control skin (Fig. 6*A*), and this finding was confirmed at the protein level in dermal fibroblasts and endothelial cells by immunohistochemistry (Fig. 6 *B* and *C*). Notably, increased *IRF5* mRNA levels correlated significantly with an increase of the *COL1A1*, *COL1A2*, and connective tissue growth factor (*CTGF*) mRNA levels in SSc lesional skin (Fig. S5*A*). Furthermore, *IRF5* mRNA levels were elevated significantly in patients with diffuse cutaneous SSc (dcSSc) as compared with patients with limited cutaneous SSc (lcSSc) and in SSc patients with a current and past history of digital ulcers as compared with patients without such a history (Fig. S5*B*). Thus, in so far as we could determine in these SSc patients, these data are consistent with the murine model of SSc.

In in vitro studies we confirmed that *IRF5* mRNA levels were elevated in SSc dermal fibroblasts as compared with normal dermal fibroblasts (Fig. S5*C*) and correlated positively with *COL1A2* mRNA levels in normal and SSc dermal fibroblasts (Fig. S5*D*). Importantly, in normal human fibroblasts IRF5 expression was increased by LPS and/or TGF-β stimulation at the mRNA and protein levels (Fig. S5*E*). Therefore IRF5 itself may be up-regulated in response to profibrotic stimuli in SSc dermal fibroblasts.

The SNP rs4728142 is located in the *IRF5* promoter, and its minor allele A is seen in 9% of Japanese population (24). Supporting the relationship between the rs4728142 A allele and less serious clinical presentation (24), *IRF5* mRNA levels in the

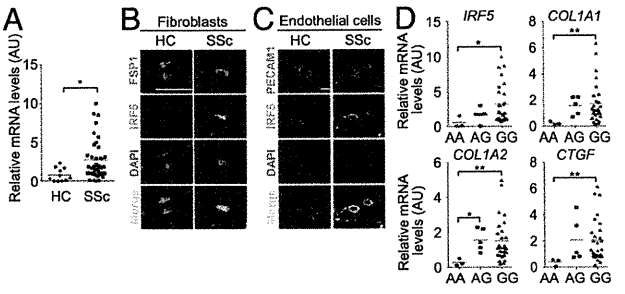

Fig. 6. IRF5 expression and its association with the profibrotic condition in SSc. (*A*) *IRF5* mRNA expression in bulk skin from healthy controls (HC) and SSc patients. (*B*) Immunofluorescence staining for FSP1 (red), IRF5 (green), and DAPI (blue) in skin samples from HC and SSc subjects (n = 4). (Scale bar, 20 μm.) (*C*) Immunofluorescence staining for platelet/endothelial cell adhesion molecule 1 (PECAM1) (red), IRF5 (green), and DAPI (blue) in skin samples from HC and SSc subjects. (Scale bar, 20 μm.) (*D*) The comparison of relative mRNA levels of the *IRF5*, *COL1A1*, *COL1A2*, and *CTGF* genes in bulk skin from SSc patients with the AA genotype, AG genotype, and GG genotype. *P < 0.05 and **P < 0.01 by two-tailed unpaired t-test.

lesional skin were observed to be significantly lower in SSc patients with the AA genotype than in those with the GG genotypes (Fig. 6*D*). Importantly, *COL1A1*, *COL1A2*, and *CTGF* mRNA levels were decreased significantly in SSc patients with the AA genotype as compared with those with the GG genotype (Fig. 6*D*). Furthermore, this allele tended to be related to milder interstitial lung disease with higher percent diffusing capacity for carbon monoxide (%DLco) (Table S1). Therefore, the rs4728142 A allele leads to a milder clinical presentation of SSc, possibly correlated with inhibited activation of fibroblasts.

Discussion

In this study we provide the first evidence, to our knowledge, of a critical role of IRF5 in the mouse model of SSc. Reflecting its critical role as a regulator of the immune system, IRF5 has been identified as a susceptibility gene in various diseases related to immune abnormalities, including SSc (14). Our study reveals that IRF5 is involved in many aspects of pathological processes (e.g., fibrotic and vascular aspects and immune cell abnormalities), as either a transcriptional activator or a repressor. Supporting this idea, IRF5 was observed to bind the promoters of various target genes in fibroblasts, endothelial cells, and lymphocytes. Of note, a direct involvement of IRF5 in immune polarization of $CD4^+$ T cells and B-cell activation was suggested also. Most importantly, loss of *Irf5* attenuated tissue fibrosis, vascular activation, and inflammation in BLM-treated mice. To the best of our knowledge, this is the first report disclosing the diverse effects of IRF5 on all three cardinal pathological features of SSc.

Innate immune signaling-mediated fibroblast activation was recently reported in SSc and its animal models. In SSc, serum levels and dermal expression levels of endogenous TLR4 ligands, such as HMGB1, hyaluronan, and fibronectin extra domain, are elevated (5, 6, 8, 9). In in vitro experiments, TLR4 signaling induced by these candidate ligands results in fibroblast activation (5, 6). TLR4 signaling also augments TGF-β signaling and, indeed, TLR4 blockade suppresses basal and TGF-β–dependent activation of fibroblasts (5, 6). Importantly, in *Tlr4*-deficient mice BLM-induced dermal fibrosis is attenuated significantly despite the elevation of endogenous TLR4 ligands (7). Taken together, TLR4 signaling appears to be involved in pathological dermal fibrosis by directly activating dermal fibroblasts. As described in this study, $Irf5^{-/-}$ dermal fibroblasts exhibited an antifibrotic phenotype under normal physiological conditions. In addition, ectopic expression of IRF5 enhanced *COL1A2* promoter activity, especially by the stimulatory signals via TGF-β1 and TLR4, both of which also increase IRF5 expression in human dermal fibroblasts. Most importantly, TLR4 activation augmented IRF5's occupancy on the *COL1A2* promoter in human dermal fibroblasts, whereas IRF5 binding to the *Col1a2* promoter was reduced remarkably in $Tlr4^{-/-}$ murine dermal fibroblasts. Therefore, the amplification of TGF-β stimulation by TLR4 signaling may be exerted, at least partially, through IRF5 activation in dermal fibroblasts. Given that the skin phenotype of $Irf5^{-/-}$ mice was largely contrary to that of human SSc at the histological and ultrastructural levels and that IRF5 was up-regulated in SSc dermal fibroblasts, we conclude IRF5 plays a pivotal role in tissue fibrosis in SSc. This notion also is supported by the significant positive correlation of *IRF5* mRNA levels with *COL1A1*, *COL1A2*, and *CTGF* mRNA levels in SSc lesional skin.

SSc vasculopathy is marked by specific structural and functional abnormalities (1). The structural changes of SSc vasculature are largely attributable to vascular instability closely related to the altered phenotype of mural cells. In SSc lesional skin, α-SMA expression is decreased in mural cells, reflecting the weak interaction of endothelial cells with mural cells and the activation of proangiogenic signaling pathways (23). This feature was reproducible in BLM-treated mice, but in $Irf5^{-/-}$ mice a high α-SMA expression was maintained in the skin vasculature even after BLM injection, indicating that IRF5 is a key regulator of the balance between angiostatic and proangiogenic conditions. Consistent with this notion, IRF5 bound the promoters of the *CDH5* and *PDGFB* genes encoding key molecules—VE-cadherin and PDGF-B, respectively—regulating vascular stabilization and angiogenesis. Because of its involvement in mechanisms connecting vascular activation and tissue fibrosis, EndoMT is a critical pathological event in human and murine models of SSc (25, 26). Because the loss of *Irf5* decreased the number of double-positive cells for FSP1 and VE-cadherin in the perivascular region of BLM-treated skin, IRF5 is implicated as a critical regulator of EndoMT, as also was confirmed by IRF5 binding to the *SNAIL1* promoter. Importantly, IRF5 occupancy on the *CDH5* promoter was enhanced by TLR4 activation. Together with the evidence that TLR4 and its endogenous ligands have been implicated in angiogenesis (27, 28), the present data provide a previously unidentified insight into the role of IRF5 in TLR4-mediated angiogenesis and suggest that the TLR4–IRF5 axis potentially regulates vascular features of SSc.

The regulation of $CD4^+$ T-cell infiltration by cell adhesion molecules has attracted much attention in the context of pathological tissue fibrosis, including SSc and its animal models (25, 29). Studies with mice deficient for cell adhesion molecules have revealed that ICAM-1 and GlyCAM-1 promote tissue fibrosis along with the infiltration of Th2/Th17 cells, macrophages, and mast cells, whereas P-selectin and E-selectin suppress tissue fibrosis accompanying Th1 cell infiltration in response to BLM (29). In lesional skin of early dcSSc, the expression levels of the profibrotic cell adhesion molecules ICAM-1 and GlyCAM-1 are increased relative to that of the antifibrotic cell adhesion molecules E-selectin and P-selectin (25). Therefore our observation that the deletion of *Irf5* reduced the relative expression of profibrotic cell adhesion molecules to antifibrotic molecules, in turn leading to the decreased infiltration of macrophages and mast cells and a Th1-predominant cytokine balance, indicates a critical role for IRF5 in the induction of profibrotic inflammation. In addition, we also demonstrated that Th1-skewed immune polarization in BLM-treated $Irf5^{-/-}$ mice is linked to IRF5 binding to the *Tbet* promoter, suggesting that IRF5 directly suppresses Th1 cell differentiation. Given that IRF5's occupancy of the *Tbet* promoter was decreased in $Tlr4^{-/-}$ $CD4^+$ T cells, the activation of TLR4–IRF5 axis promotes a Th2/Th17-predominant inflammatory condition through the direct suppression of Th1 cell differentiation, which may be characteristic of SSc (1).

B-cell activation also is an important part of immune abnormalities in SSc (22). In addition to fibroblasts, endothelial cells, and T cells, deletion of *Irf5* induced the phenotypical alteration of B cells, such as down-regulation of CD19 and decreased IL-6 release, a state contrary to SSc B cells. Furthermore, IRF5 bound the *Cd19* and *Il6* promoters in murine B cells, and occupancy of the *Il6* promoter was decreased in $Tlr4^{-/-}$ B cells and increased in $Tlr4^{+/+}$ B cells upon TLR4 activation. Therefore, the TLR4–IRF5 axis directly regulates B-cell activation. Given that *Il6* mRNA levels were decreased markedly in the skin of BLM-treated $Irf5^{-/-}$ mice and that IL-6 is a classic inflammatory cytokine produced by various cells, we surmise that IRF5 regulates IL-6 production in other cell types. Because IL-6 enhances collagen synthesis by inducing myofibroblastic differentiation, promotes Th2 and Th17 differentiation, and inhibits Th1 polarization (30, 31), the loss of IL-6 production largely contributes to the attenuation of tissue fibrosis in BLM-treated $Irf5^{-/-}$ mice.

Based on data in mice, it is assumed that up-regulated expression of IRF5 is involved in the development of three cardinal features of SSc. Supporting this idea, IRF5 expression was increased in dermal fibroblasts and vascular cells in the lesional skin of SSc patients. Furthermore, *IRF5* mRNA levels correlated positively with *COL1A1*, *COL1A2*, and *CTGF* mRNA levels in

SSc dermal fibroblasts. Moreover, as is consistent with the linking of rs4728142 A allele to a milder clinical phenotype resulting from the loss of IRF5 induction, SSc dermal fibroblasts with the AA genotype expressed *COL1A1*, *COL1A2*, and *CTGF* mRNA at lower levels than SSc dermal fibroblasts with the GG genotype. Reflecting the role of IRF5 in SSc vasculopathy, SSc patients with a current and past history of digital ulcers had higher *IRF5* mRNA levels than patients with no history of digital ulcers. Given that SSc progresses via a complex interaction between fibroblasts, endothelial cells, and immune cells with SSc-specific phenotypes, the attenuation of SSc phenotypes in each of those cells resulting from IRF5 down-regulation appears to result in milder clinical symptoms of this disease. To our knowledge, these are the first data providing a molecular basis for an association of the rs4728142 A allele with a milder SSc phenotype.

In summary, we provide evidence that IRF5 is involved in the three cardinal pathological processes of SSc through the direct transcriptional regulation of target genes in various cell types. In particular, it is interesting that IRF5 could serve as a potent repressor for the *Tbet* gene, because IRF5 acts primarily as a potent inducer of various immune-related and inflammatory genes (17). Also, contrary to our previous findings on serum MMP-13 levels and endothelial VE-cadherin expression in SSc (23, 32), *Irf5* deficiency induced *Mmp13* gene expression in dermal fibroblasts and *Cdh5* gene expression in endothelial cells, indicating a role for IRF5 as a transcriptional repressor in various types of cells. In this regard, it may be worth recalling that such a bifunctional action has been shown for IRF3 (20). Obviously, further work is required to elucidate the underlying mechanisms by which IRF5 exerts a repressive function. Importantly, our study revealed the contribution of innate immune signaling via the TLR4–IRF5 axis to the induction of SSc phenotypes in various cell types, offering clues for further understanding of the roles of environmental factors and endogenous ligands in the development and persistence of disease. Finally, our study may provide impetus for new drug development by targeting IRF5 in the treatment of SSc.

Methods

All experimental procedures were approved by the ethical committee of University of Tokyo Graduate School of Medicine. Written informed consent was obtained from all patients and healthy individuals. The study was performed according to the Declaration of Helsinki. All animal studies and procedures were approved by the Committee on Animal Experimentation of University of Tokyo Graduate School of Medicine. Generation of BLM-treated mice, histological assessment, the hydroxyproline assay, transmission electron microscopy, immunoblotting, RNA isolation, quantitative RT-PCR (qRT-PCR), ChIP, the oligonucleotide pull-down assay, the reporter assay, flow cytometric analysis, the vascular permeability assay, immunofluorescence, cell culture, plasmid information, and the SNP genotyping assay are described in *SI Methods*. Primer sequences for qRT-PCR and ChIP are summarized in Tables S2 and S3, respectively.

Mice. C57BL/6 female mice (6 to 8 wk old) were used in this study. *Irf5*$^{-/-}$ mice were described previously (17). *Tlr4*$^{-/-}$ mice and tight skin mice were purchased from The Jackson Laboratory.

Patients. cDNA and genomic DNA were prepared from forearm skin samples of 36 SSc patients [33 females; median age (25–75 percentile): 57 y; range 41.0–67.0 y] and 11 healthy donors (8 females; median age, 50 y; range 38.0–69.0 y). Fibroblasts were obtained by skin biopsy from eight sex- and site-matched SSc patients and eight healthy donors of closely matched age.

Statistical Analysis. Statistical analysis was carried out with one-way ANOVA with Bonferroni post hoc tests for multiple group comparisons and the two-tailed unpaired *t*-test for two group comparisons. For comparing two group values that did not follow Gaussian distribution, the two-tailed Mann–Whitney *u* test was used. $P < 0.05$ was considered statistically significant. Within-group distributions are expressed as mean ± SEM.

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Tamibarotene ameliorates bleomycin-induced dermal fibrosis by modulating phenotypes of fibroblasts, endothelial cells, and immune cells

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Short title: Am80 ameliorates BLM-induced dermal fibrosis

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Abbreviations: BLM, bleomycin; SSc, systemic sclerosis; ATRA, all-*trans* retinoic acid; NO, nitric oxide; EndoMT, endothelial-to-mesenchymal transition; WT, wild type; PBS, phosphate-buffered saline; H&E, hematoxylin and eosin; qRT-PCR, quantitative real-time reverse transcription polymerase chain reaction; HDMECs, human dermal microvascular

endothelial cells; MFI, mean fluorescence intensity; TGF-β1, transforming growth factor-β1; CTGF, connective tissue growth factor; TNF-α, tumor necrosis factor-α; CCL2, chemokine (C-C motif) ligand 2; MCP-1, monocyte chemotactic protein 1; IFN, interferon; FSP1, fibroblast-specific protein 1; TSK1, tight skin 1; ICAM-1, intercellular adhesion molecule-1

Abstract

Tamibarotene (Am80) is a synthetic retinoid that modulates the pathological processes of various autoimmune and inflammatory diseases and their animal models. We here investigated the therapeutic potential of Am80 against systemic sclerosis (SSc) utilizing its animal models. Am80 significantly attenuated dermal and hypodermal fibrosis in bleomycin (BLM)-treated mice and tight skin 1 mice, respectively. Consistently, Am80 significantly suppressed the expression of various molecules related to tissue fibrosis, including TGF-β1, CTGF, IL-4, IL-10, IL-13, IL-17A, TNF-α, IFN-γ, and MCP-1, in the lesional skin of BLM-treated mice. Furthermore, Am80 decreased the proportion of effector T cells, while increasing that of naïve T cells among $CD4^+$ T cells in the draining lymph nodes of BLM-treated mice. Moreover, a series of BLM-induced pathological events, including endothelial-to-mesenchymal transition, ICAM-1 expression in endothelial cells, the infiltration of macrophages, mast cells, and lymphocytes, and M2 macrophage differentiation, were attenuated by Am80. Importantly, Am80 directly reversed the pro-fibrotic phenotype of TGF-β1-treated dermal fibroblasts, suppressed ICAM-1 expression in endothelial cells, and promoted M1 macrophage differentiation *in vitro*. Collectively, Am80 inhibits the development of experimental dermal fibrosis by reversing the pro-fibrotic phenotype of various cell types and would be a candidate for new therapeutic drugs against dermal fibrosis of SSc.

INTRODUCTION

Systemic sclerosis (SSc) is a multisystem autoimmune disease characterized by initial vascular injury and resultant fibrosis of the skin and various internal organs (LeRoy *et al.*, 1988). Although the pathogenesis of SSc still remains unclear, some growth factors, cytokines, chemokines, and cell adhesion molecules have been implicated in its developmental processes (Asano and Sato, 2015). Currently, the choice and efficacy of treatment are limited, and the development of new therapies awaits the elucidation of disease etiology.

Several retinoids have been developed and applied in clinical settings, including etretinate, acitretin, tazarotene, tretinoin (all *trans* retinoic acid [ATRA]), and tamibarotene (Am80). Am80 is an agonist for retinoic acid receptor α/β, which is primarily intended for the treatment of acute promyelocytic leukemia relapsed from or refractory to ATRA. Am80 has chemical and pharmacological advantages over ATRA, including higher chemical stability, lower affinity for cellular retinoic acid-binding protein, and a lack of affinity for retinoic acid receptor-γ (Miwako and Kagechika, 2007). Since retinoic acid receptor-γ is a positive regulator of inflammatory cytokine production in macrophages and $CD8^+$ T cells, Am80 may be better than other retinoids in ameliorating some inflammatory conditions (Dzhagalov *et al.*, 2007). Consistently, some effects of Am80 on the immune system have been reported. For instance, Am80 attenuates neutrophil migration and reactive oxygen species production and elastase release from those cells in a murine vasculitis model and/or human cultivated neutrophils (Miyabe *et al.*, 2013). In experimental autoimmune myositis, Am80 enhances the differentiation into T helper 1 (Th1) and T helper 2 (Th2) cells, but not T helper 17 (Th17) cells, while suppressing the infiltration of inflammatory cells, pro-inflammatory cytokine production, and antibody production (Ohyanagi *et al.*, 2009). Furthermore, Am80 promotes the differentiation

into Th1 cells, while inhibiting the differentiation into Th17 cells or regulatory T cells in murine collagen-induced arthritis (Sato *et al.*, 2010). On the other hand, retinoids also have beneficial effects on vascular disorders. For example, ATRA ameliorates atherosclerosis by inducing the differentiation and inhibiting the proliferation of vascular smooth muscle cells (Miano and Berk, 2000, 2001). As for Am80, this agent inhibits angiogenesis caused by the interaction between endothelial cells and myeloma cells *in vitro* (Sanda *et al.*, 2005). Thus, Am80 diversely modulates the pathological immune and vascular processes in the context-dependent manner.

Given that aberrant vascular and immune activation eventually leads to fibroblast activation in SSc, Am80 may have a potential therapeutic effect on SSc by modulating vascular, immunological, and fibrotic aspects. To assess this hypothesis, we here investigated the effect of Am80 on dermal fibrosis of a bleomycin (BLM)-induced murine model of SSc and tight skin 1 (TSK1) mice by focusing on the three major pathological components of SSc, fibrosis, vasculopathy, and immune abnormalities.

RESULTS

Am80 alleviates skin fibrosis in BLM-induced SSc model mice and TSK1 mice.

Dermal thickness of lesional skin sections was greater in BLM-treated mice than in phosphate-buffered saline (PBS)-treated mice on day 28, while comparable on day 7. Of note, Am80 significantly attenuated BLM-induced dermal thickness on day 28 (Figure 1a). Consistent with this finding, Am80 treatment significantly reduced collagen content and mRNA expression of the *Col1a1, Col1a2, Col3a1,* and *Col5a1 genes*, while promoting that of the *Mmp13* gene in the lesional skin of BLM-treated mice (Figure 1b and 1c). Since transforming growth factor (TGF)-β1 and connective tissue growth factor (CTGF) are enough to induce experimental dermal

fibrosis and their elevated expression is the hallmark of SSc dermal fibroblasts (Chujo *et al.*, 2005; Noda *et al.*, 2014), we further examined the expression of these growth factors. As expected, the decrease in mRNA expression of the *Tgfb1* and *Ctgf* genes was noted in BLM-treated mice exposed to Am80 (Figure 1d), which was also confirmed at protein levels by immunohistochemistry (Figure 1e). To further elucidate the anti-fibrotic effect of Am80 on tissue fibrosis, we employed TSK1 mice, another widely accepted murine SSc model characterized by increased hypodermal thickness (Green *et al.*, 1976). In line with the results of BLM-treated mice, Am80 significantly ameliorated hypodermal thickness and collagen content in the back skin of TSK1 mice (Figure 1f and 1g). These findings suggest that Am80 exerts a potent anti-fibrotic effect on dermal fibrosis by reducing the production of collagen, promoting its degradation, and regulating expression of various soluble factors in SSc murine models.

Am80 inhibits the production of IL-4, IL-17A, and IFN-γ in $CD4^+$ T cells.

To investigate the impact of Am80 on BLM-induced inflammatory condition, we next examined the expression of pro-inflammatory and T helper cytokines and chemokines, including tumor necrosis factor (TNF)-α, monocyte chemotactic protein 1 (MCP-1), interferon (IFN)-γ, interleukin (IL)-4, IL-6, IL-10, IL-13, and IL-17A in the lesional skin of BLM-treated mice. Notably, a significant reduction of mRNA expression due to Am80 treatment was seen in all the genes except *Il6* (Figure 2a), indicating that Am80 widely suppresses the expression of pro-inflammatory, Th1, Th2, and Th17 cytokines, and chemokines in the context of BLM-treated murine skin. To further assess if Am80 diversely prevents the differentiation of T helper cells, we further carried out flow cytometric analysis with draining lymph nodes. Of note, Am80 significantly restored the BLM-dependent increase in the total lymphocyte number in draining

lymph nodes (Figure 2b). More importantly, draining lymph nodes from BLM-treated mice administered Am80 included the smaller number of Th1, Th2, and Th17 cells, which was evaluated by cytokine production and the expression of master regulators of differentiation, and $Foxp3^{+}CD25^{+}CD4^{+}$ regulatory T cells than draining lymph nodes from BLM-treated mice without the exposure to Am80 (Figure 2c and 2d). In agreement with these findings, Am80 significantly increased the proportion of naïve T cells ($CD62L^{+}CD44^{low}$ cells), while decreasing that of effector memory T cells ($CD62L^{-}CD44^{high}$ cells) in $CD4^{+}$ T cells (Figure 2e). Thus, Am80 inhibits differentiation of naïve $CD4^{+}$ T cells into cytokine-producing effector memory T cells in BLM-treated mice.

Am80 suppresses M2 polarization of macrophages.

Since M2 macrophages are involved in the pathological fibrotic response, we also evaluated the impact of Am80 on macrophages in the context of BLM-treated mice. In accordance with its anti-fibrotic property as described above, Am80 significantly reduced mRNA expression of *Ym1* and *Fizz1*, established polarization markers for M2 macrophages, in the skin lesions of BLM-treated mice (Figure 3a). *In vitro*, Am80 also significantly reversed the IL-4-dependent induction of *Ym1* and *Fizz1* mRNA expression and the IL-10 and TGF-β1-dependent induction of cell surface markers of M2 macrophages, including CD204 and CD206, in peritoneal macrophages (Figure 3b - 3d). In contrast, the expression levels of M1 macrophage markers, INOS and IL-12p35, were enhanced in response to Am80 in the lesional skin of BLM-treated mice and in THP-1 cells stimulated with IFN-γ and lipopolysaccharides (Figure 3e and 3f). Taken together, these results indicate that Am80 promotes differentiation of M1 macrophages *in vivo* and *in vitro*.